Stress, Solvent Production & Tolerance (in Clostridium acetobutylicum)

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Why?

- Solvent (and toxic chemical) tolerance in crucial for production of chemicals, bioremediation, whole-cell biocatalysis. But, ALSO, crucial basic knowledge: How do organisms adapt to "toxic" environments?
- Can we use ME (and genomic approaches) for targeted genetic changes to generate more tolerant strains for bioprocessing?
- Past efforts to produce tolerant strains have relied on selection under applied pressure and chemical mutagenesis: some good results, but not always consistent. Can we do better?

What constitutes solvent toxicity? Tolerance?

- Accepted dogma. Toxicity is due to chaotropic effects of solvents on cell membrane: impaired membrane fluidity and functions (nutrient transport, energy metabolism, ion transport) inhibit cell metabolism, and result in cell death
- THUS, tolerance is ability of membrane to withstand high levels of toxic chemicals without loss of "function": different membrane composition (and perhaps membrane proteins?)
- Some organisms tolerate solvents better than others (e.g., EtOH tolerance of some lactobacilli). WHY?

Is this model sufficient for solvent production tolerance?

• Perhaps not: Several well-defined genetic modifications impart solvent tolerance (by 40-70%) without strain selection (Butyrate kinase knockout, SolR knockout): We may need to re-examine the accepted dogma

OBJECTIVE

Identify genes that may be also contributing to solvent tolerance and using genetic modifications (involving these genes) to generate solvent tolerant strains

STRATEGY

1) Overexpress stress response genes: do they impart improved solvent production (& tolerance?).

HYPOTHESIS: stress proteins are chaperonins folding/refolding/stabilizing labile proteins.

Loss of activity of such proteins may inhibit cell functions and ability to produce solvents

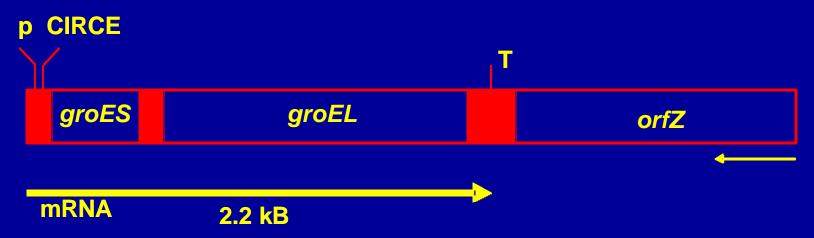
- 2) IDENTIFY classes of genes that may play an important role in solvent tolerance or toxicity
 - DNA arrays(stress genes should be included...)

Solvent Toxicity in C. acetobutylicum

HYPOTHESIS

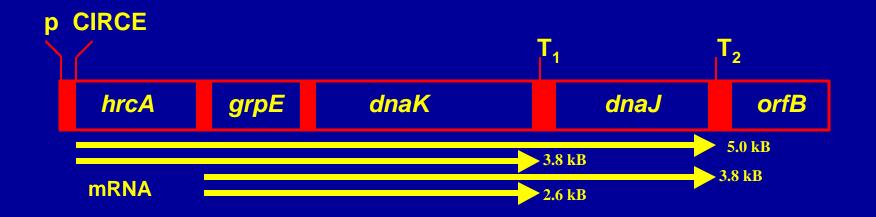
- •A potential mechanism to overcome solvent toxicity is through the over-expression of heat shock proteins, possibly providing increased protein stability
- *C. acetobutylicum* 824(pGROE1), over-expressing the molecular chaperone genes *gro*ES and *gro*EL under control of the clostridial thiolase promoter, was created to examine this hypothesis.

GroESL Operon



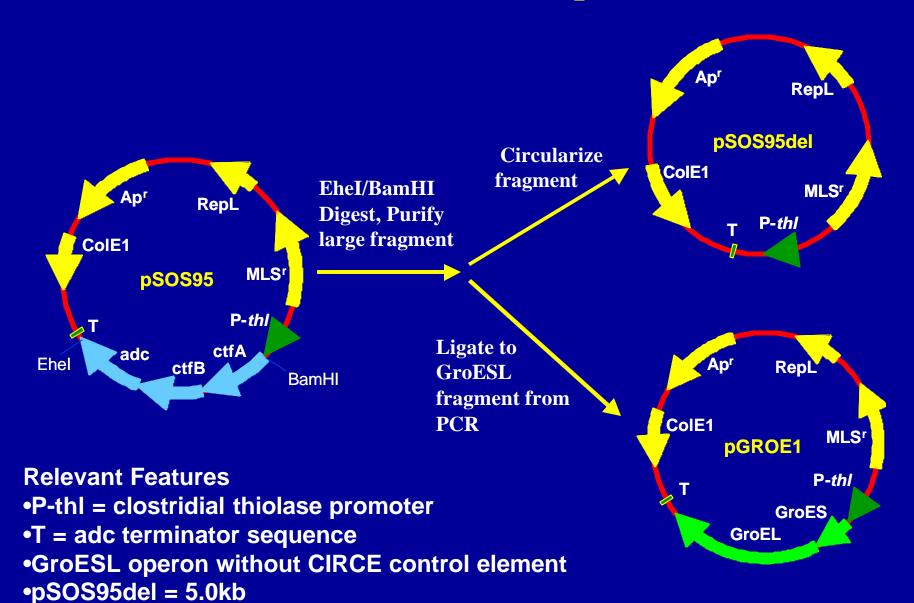
- •The GroESL operon consists of the heat shock proteins groES and groEL, a promoter (p), and a CIRCE (Controlling Inverted Repeat of Chaperonin Expression) element
- •The CIRCE element is a binding site for HrcA, a negative regulator of expression for GroESL and DnaKJ
- •HrcA is stabilized by GroES and GroEL, providing negative feedback regulation

DnaKJ Operon



•The DnaKJ operon consists of the heat shock proteins *grpE*, *dnaK* and *dnaJ*, and *hrcA* which encodes for a negative regulator of the DnaKJ and GroESL operons through binding to the CIRCE (Controlling Inverted Repeat of Chaperonin Expression) element.

Construction of GroESL Overexpression Plasmid



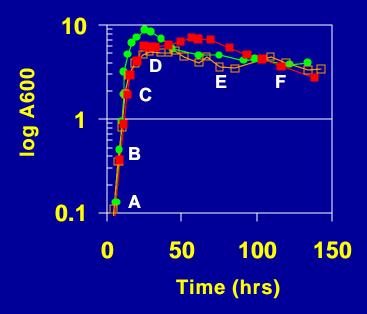
•pGROE1 = 7.1kb

Fermentation Studies

- Performed in duplicate (1.5 and 4.0 liter)
- Product concentrations measured by HPLC
- •RNA samples taken with Trizol Reagent during exponential and early stationary phases for DNA-array analysis
- Protein samples isolated through entire course of fermentation for Western analysis

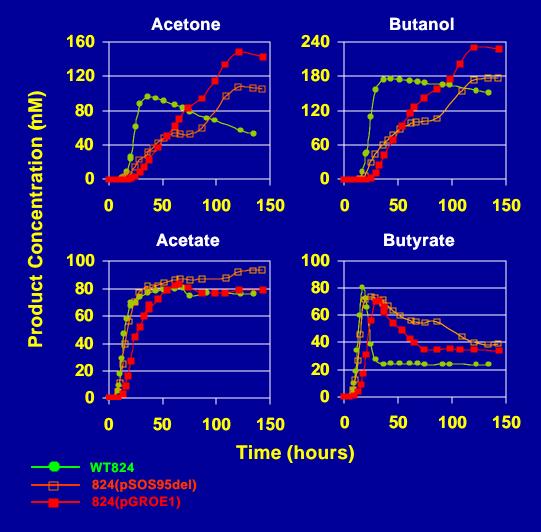
Fermentation Profiles

Fermentation Growth Curves and Sample Points



•RNA samples taken at points A, B, C and D
•Western samples taken at points B, D, E and F

Fermentation Product Profiles

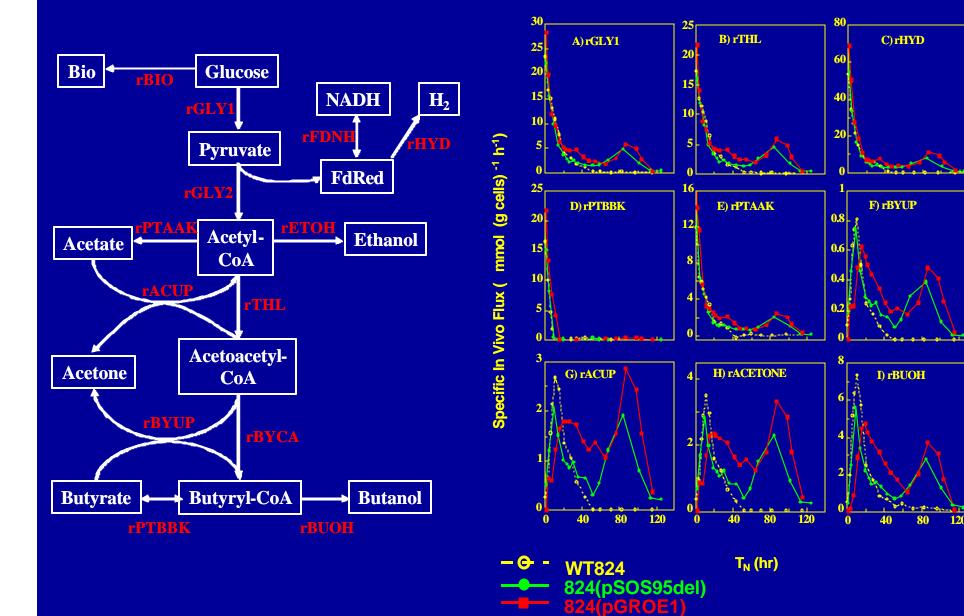


Product Concentrations

	Wild Type 824	824 (pSOS95del)	824(pGROE1)
Acetone, mM (±3)	96	107	148
Butanol, mM (±2)	175	178	231
Ethanol, mM (±1)	28	23	21
Acetate, mM (±7)	80	93	83
Butyrate, mM (±3)	80	73	70
Acetoin, mM (±1.1)	11.6	5.2	7.8
Max. O.D. (A600)	8.88	5.38	7.18
Doubling time, (hrs)	1.24	2.01	1.99

(± S.D.)

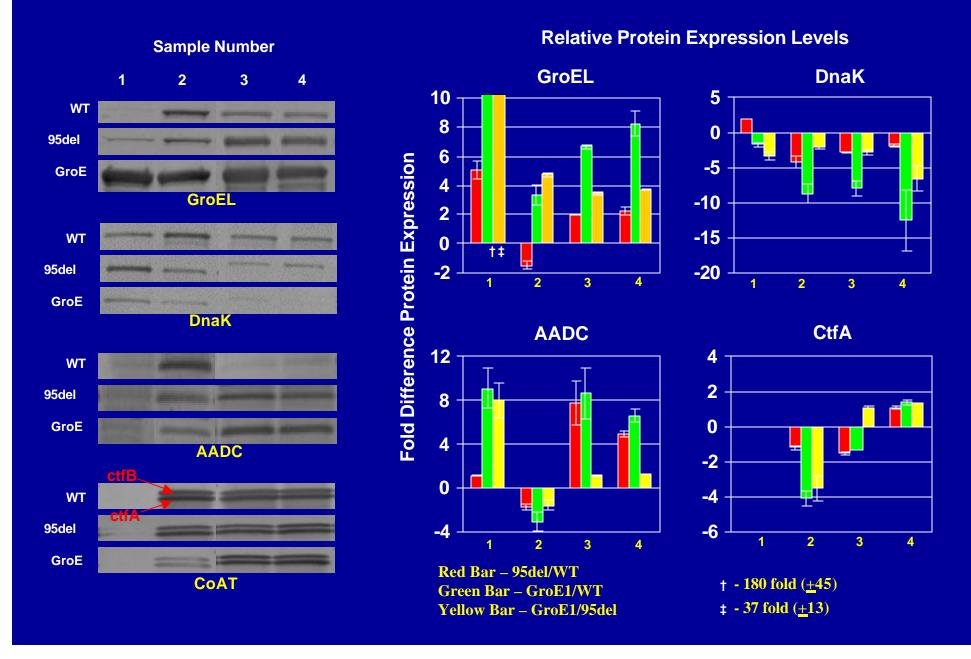
Metabolic Flux Analysis



Metabolic Flux Analysis

- Increased acetone and butanol formation fluxes (rACETONE and rBUOH)
- Increased butyrate and acetate uptake, despite higher final butyrate and acetate levels
- •824(pSOS95del) and 824(pGROE1) have very similar central metabolic pathway fluxes (rGLY1 and rTHL) and hydrogen formation (rHYD)
- •Metabolic Flux Analysis program from Desai et al., 1999

Western Analysis of Key Proteins

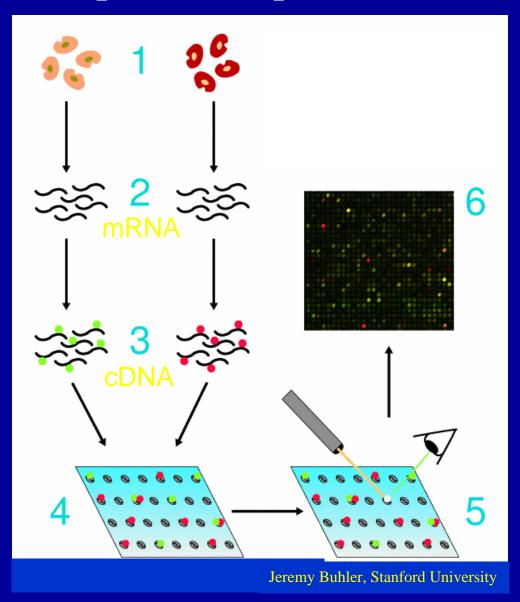


Western Analysis of Key Proteins

- GroEL Western blot confirms increased expression of GroEL at the protein level
- •Decreased expression of DnaK lends support to the concept that GroES and GroEL stabilizes HrcA, thereby resulting in decreased expression of DnaK
- •Increased AADC and CoAT levels at later time points correlates well with differential solvent production
- Increased GroES and GroEL levels may help stabilize
 AADC and CoAT during the later stages of culture

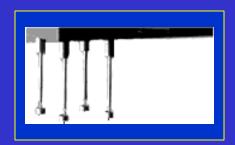
DNA-array analysis

DNA microarrays give comparative expression data

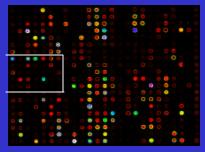


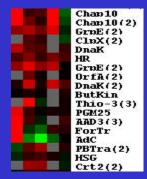
Expression analysis of *C. acetobutylicum*

- PCR genes from *C. acetobutylicum* genome
- Spot genes on aminosilane-coated glass slides with pin-and-ring arrayer
- Isolate mRNA and label cDNA from control and experimental condition with Cy3-dUTP or Cy5-dUTP
- Hybridize and scan at 550 and 650 nm
- Analyze expression and cluster genes

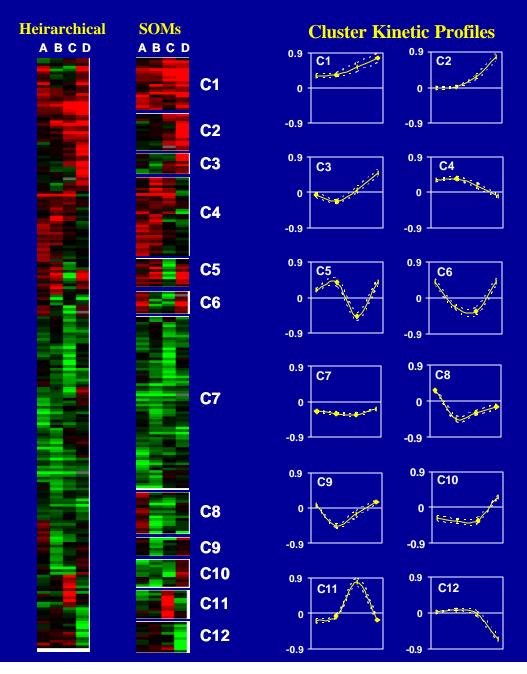






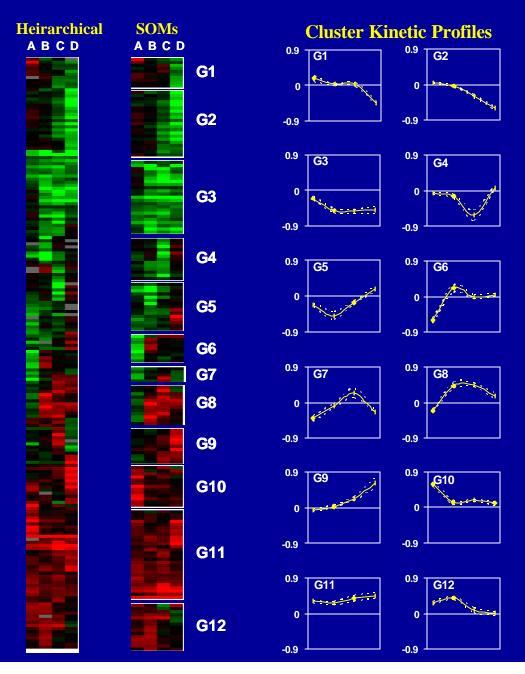


WT vs. 824(pSOS95del) Hierarchical Clustering and SOM Analysis



- 217 differentially expressed genes at the 95% confidence level
- •SOM clustering slightly more stable than the hierarchical method
- •SOM analysis resulted in 12 distinct clusters with unique gene expression patterns

824(pSOS95del) vs. 824(pGROE1) Hierarchical Clustering and SOM Analysis



- •175 differentially expressed genes at the 95% confidence level
- •SOM analysis resulted in 12 distinct clusters with unique gene expression patterns

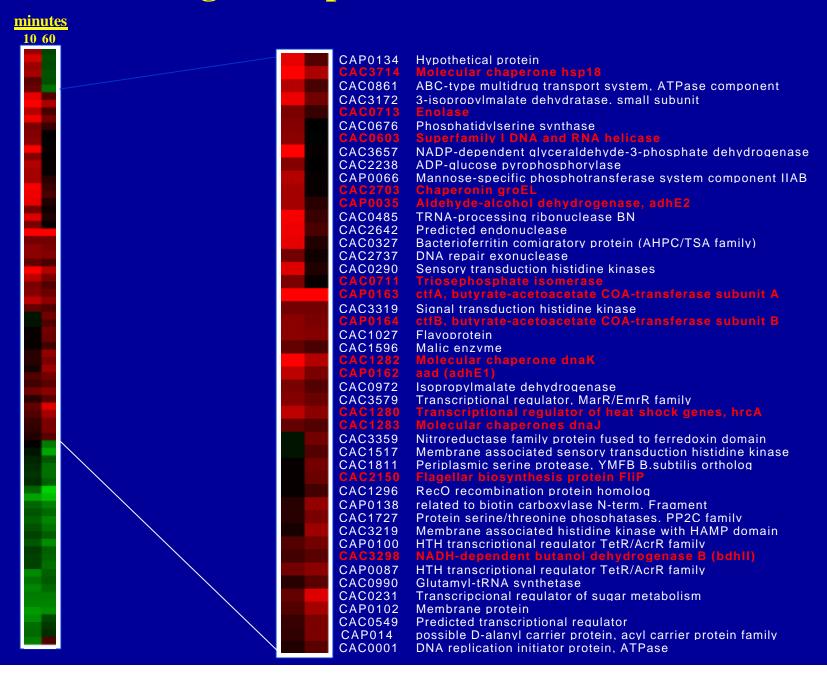
Conclusions

- •Over-expression of the GroESL operon results in increased solvent production relative to the plasmid control strain
- Increased levels of GroES and GroEL may provide added stability to proteins involved in solvent production and/or other cellular functions
- •The presence of a plasmid appears to result in a generalized stress response, helping to confirm a long standing hypothesis regarding the effects of plasmids on *C. acetobutylicum*
- •DNA-array analysis supports observations of decreased motility in plasmid control strains and suggests that over-expression of GroESL may result in a reversal of this phenotype
- •Both recombinant strains exhibit <u>sustained and</u> <u>prolonged solvent production</u>

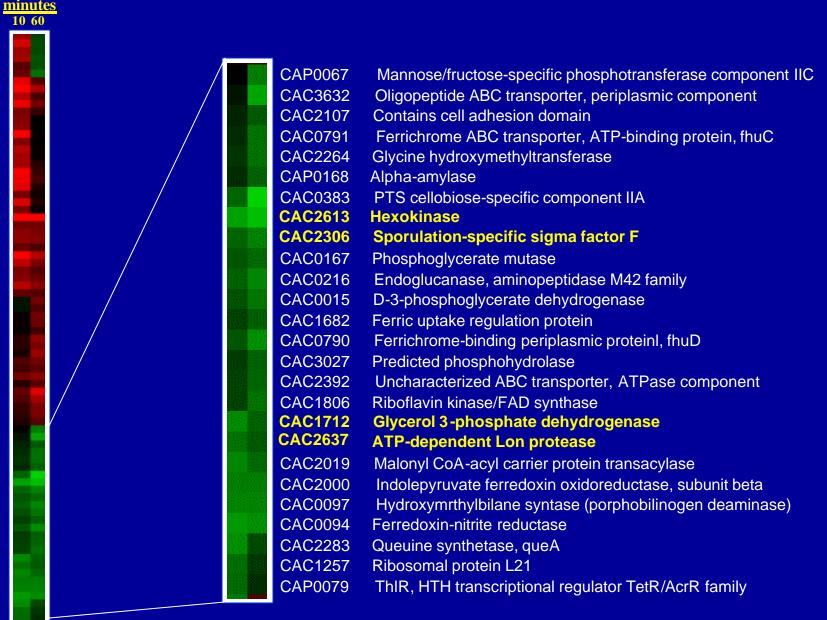
What genes are affected when cells are stressed by BuOH addition?

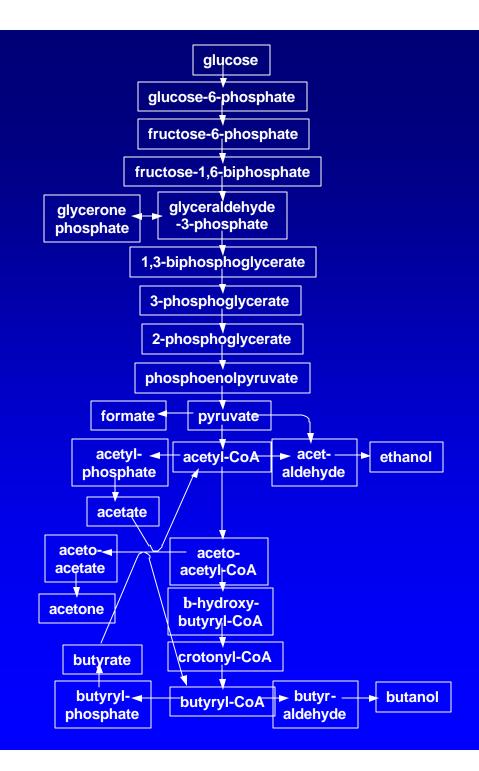
- Complex issue
- Depends on when cells are exposed to stress
- There is adaptation
- Also related to differentiation
- Several detailed protocols have been examined
- Some data are shown next

Genes with Higher Expression in BuOH Stressed Culture



Genes with Lower Expression in BuOH Stressed Culture





Patent applications

- Papoutsakis, E. T, C. Tomas, M. Tesic, and J. Y. Santiago "Increased cell resistance to toxic organic substances', US Patent No. 10/186,335 (filed 6/27/02).
- Bonarius, H, and E. T. Papoutsakis. "Increased Production of Solvents by Oxygen-Stress of Anaerobic, Solventogenic Clostridia". US provisional patent filed on December 23,2002.